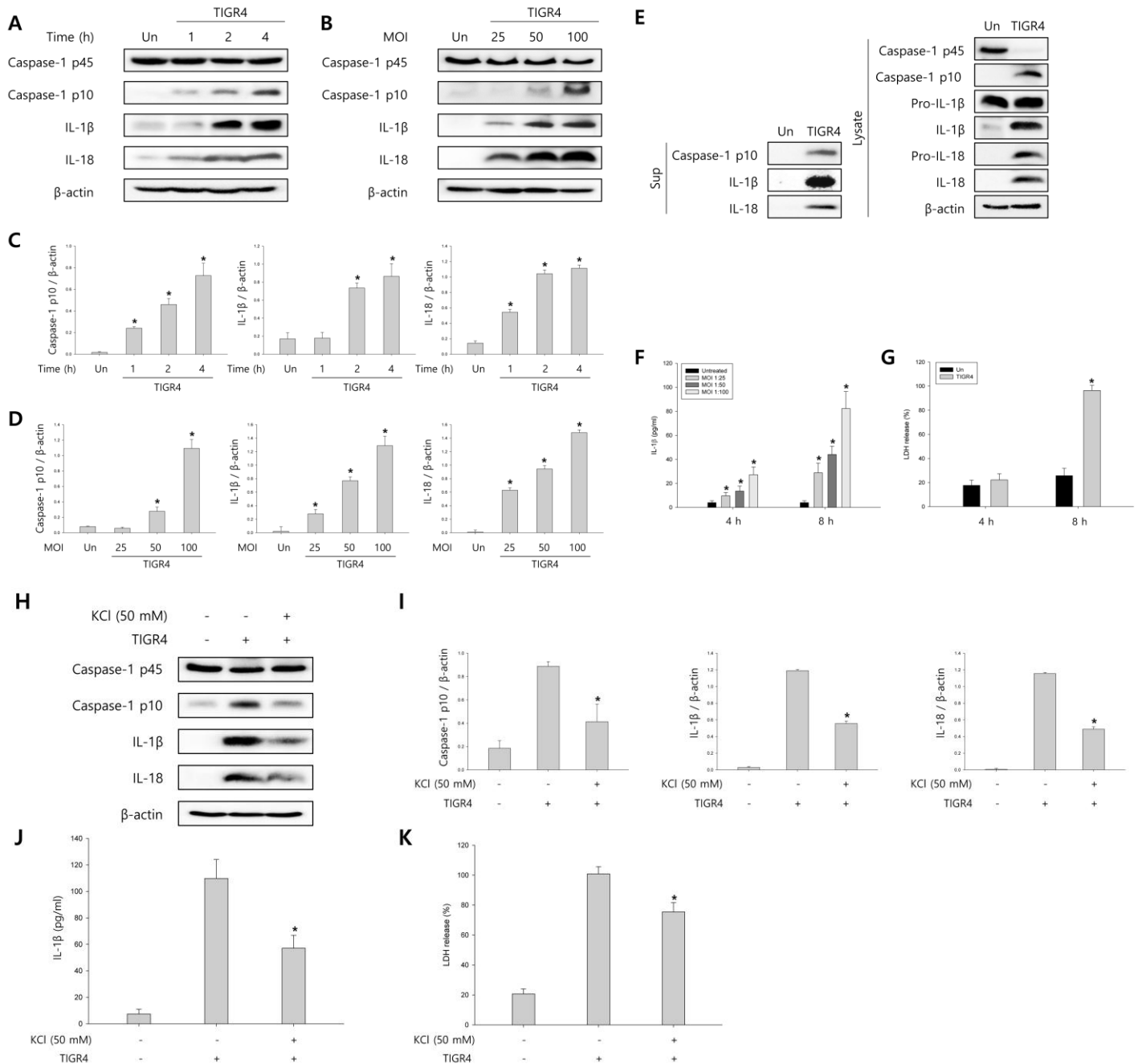


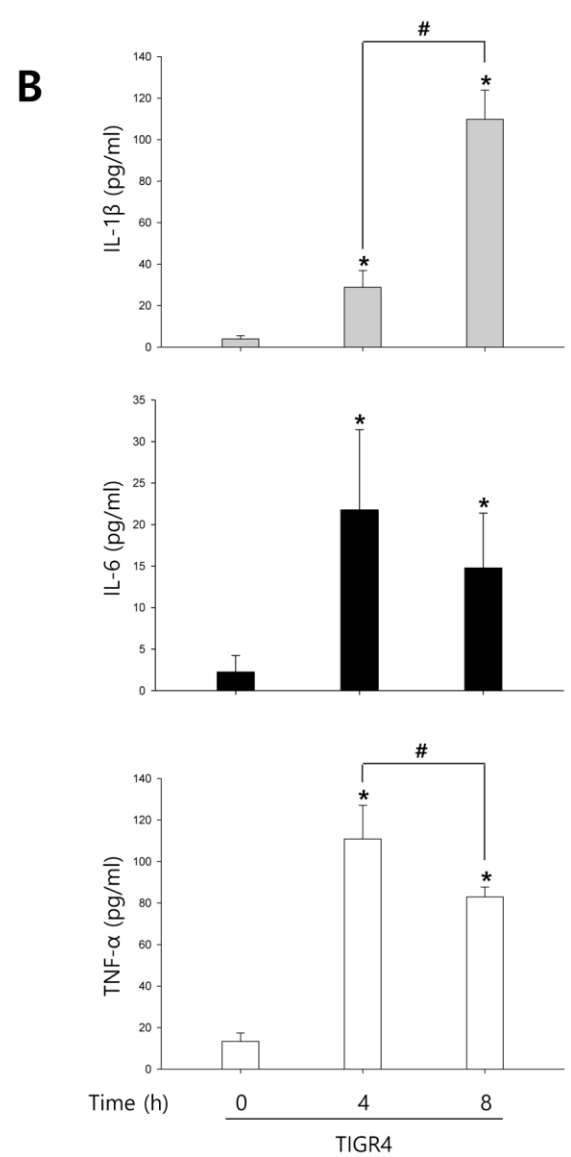
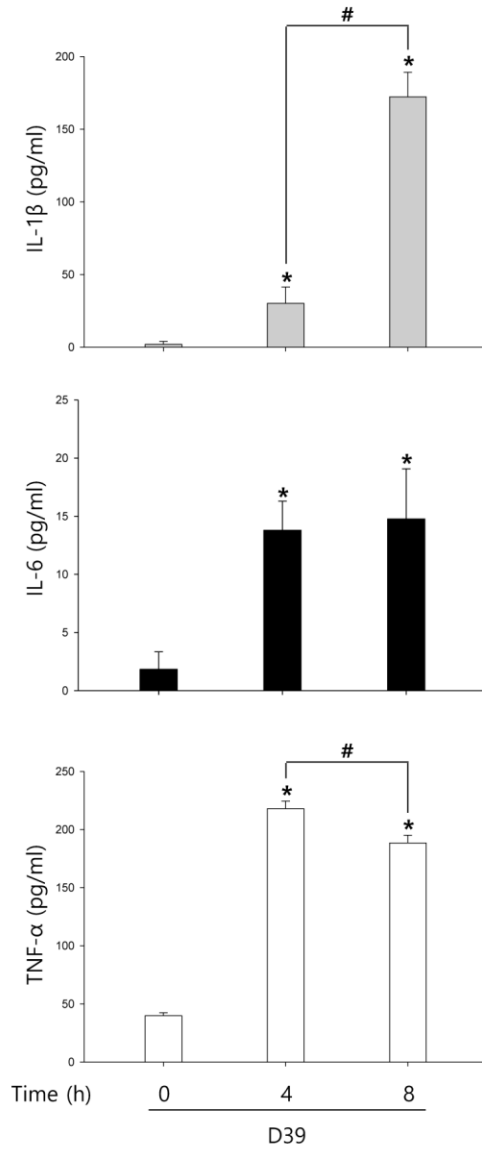
Streptococcus pneumoniae induces pyroptosis through the regulation of autophagy in murine microglia

Supplementary Material

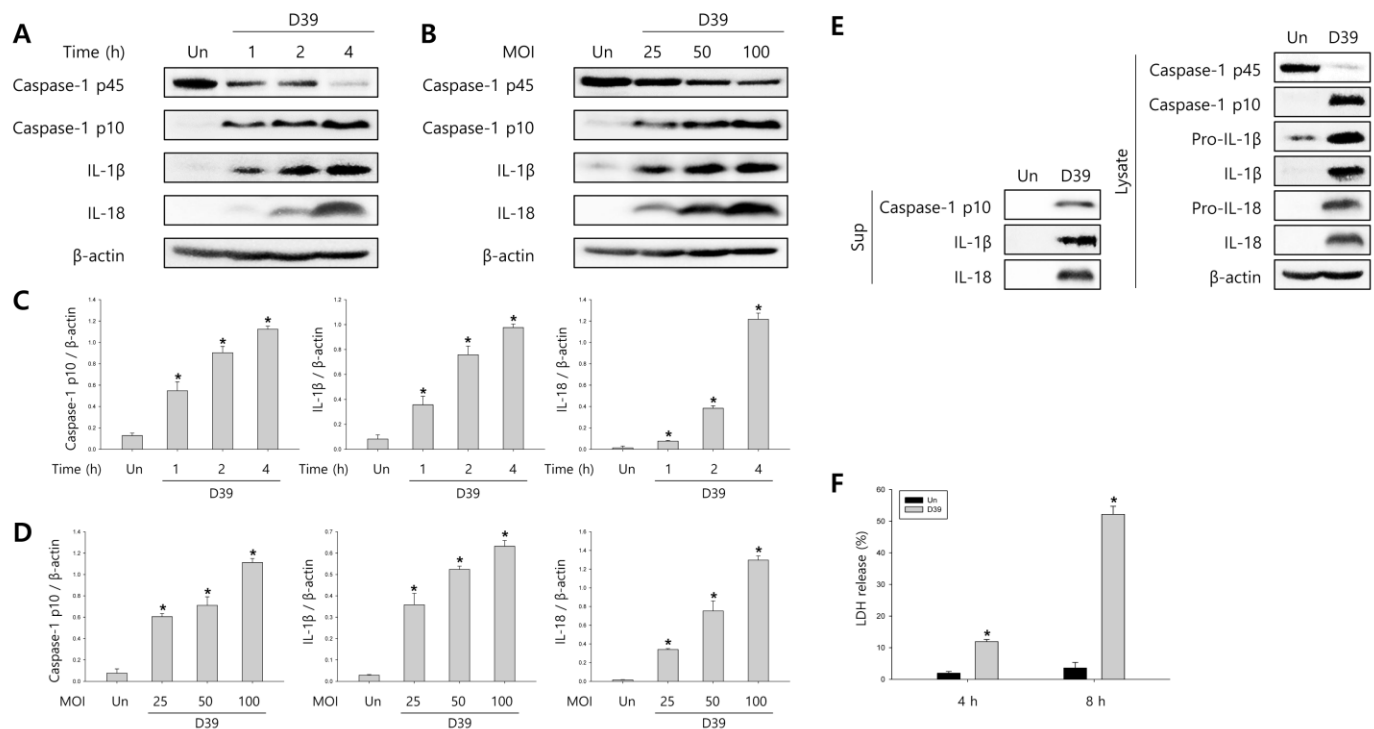


Supplementary Figure S1: *S. pneumoniae* TIGR4 induces caspase-1 activation and pyroptosis in BV-2 microglial cells. (A-D) BV-2 microglial cells were left uninfected or were infected with *S. pneumoniae* TIGR4 at various MOI of 25, 50 and 100 for 1, 2 and 4 h. Cell lysates were subjected to Western blot analysis to detect caspase-1, IL-1β and IL-18. The levels of each protein expression are in arbitrary units, and data are normalized to the respective amount of β-actin protein. These results are representative of three independent experiments with similar results. *p < 0.05, significantly different from uninfected control. (E) BV-2 cells were left uninfected or were infected with TIGR4 at an MOI of 100 for 4 h. Culture

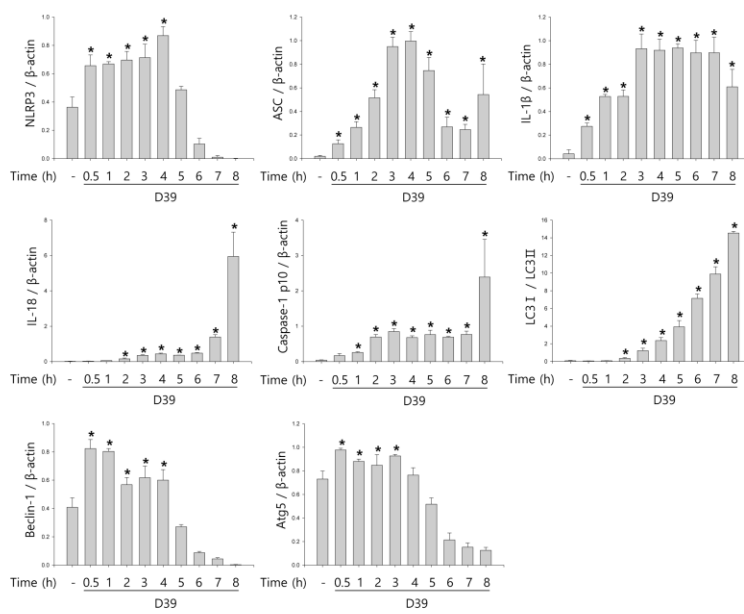
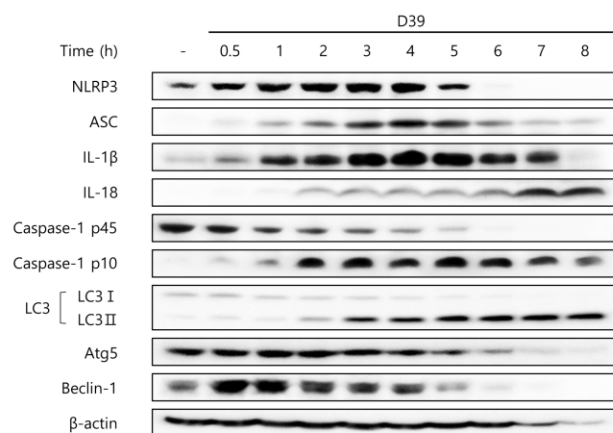
supernatants were concentrated by trichloroacetate precipitation. Concentrated supernatants and cell lysates were subjected to Western blot analysis using antibodies specific for caspase-1, IL-1 β , IL-18 and β -actin. β -actin was used as a loading control. These results are representative of three independent experiments. **(F)** Microglial cells were infected with TIGR4 at various MOI of 25, 50 and 100 for 4 h and 8 h. The levels of IL-1 β in the culture supernatants were determined by ELISA. Data are expressed as the mean \pm S.E.M. of 3 experiments. * $p < 0.05$, significantly different from uninfected control. **(G)** BV-2 microglial cells were infected with TIGR4 at an MOI of 100 for 4 h and 8 h. LDH release into the culture media is shown as a percentage of LDH release. Data are expressed as the mean \pm S.E.M. of 3 experiments. *Significantly different from uninfected cells ($P < 0.05$). **(H-K)** Potassium efflux is required for *S. pneumoniae*-induced pyroptosis. **(H and I)** BV-2 microglial cells were infected with *S. pneumoniae* TIGR4 at an MOI of 100 in the presence or absence of 50 mM of KCl for 4 h. Cell lysates were subjected to Western blot analysis to detect caspase-1 and caspase-1-dependent cytokines. The levels of each protein expression are in arbitrary units, and data are normalized to the respective amount of β -actin protein. These results are representative of three independent experiments with similar results. * $p < 0.05$, significantly different from *S. pneumoniae*-infected control. **(J and K)** BV-2 cells were infected with TIGR4 at an MOI of 100 in the presence or absence of 50 mM of KCl for 8 h. The levels of IL-1 β in the culture supernatants were determined by ELISA **(J)**. LDH release into the culture media is shown as a percentage of LDH release **(K)**. Data are expressed as the mean \pm S.E.M. of 3 experiments. * $p < 0.05$, significantly different from *S. pneumoniae*-infected control.



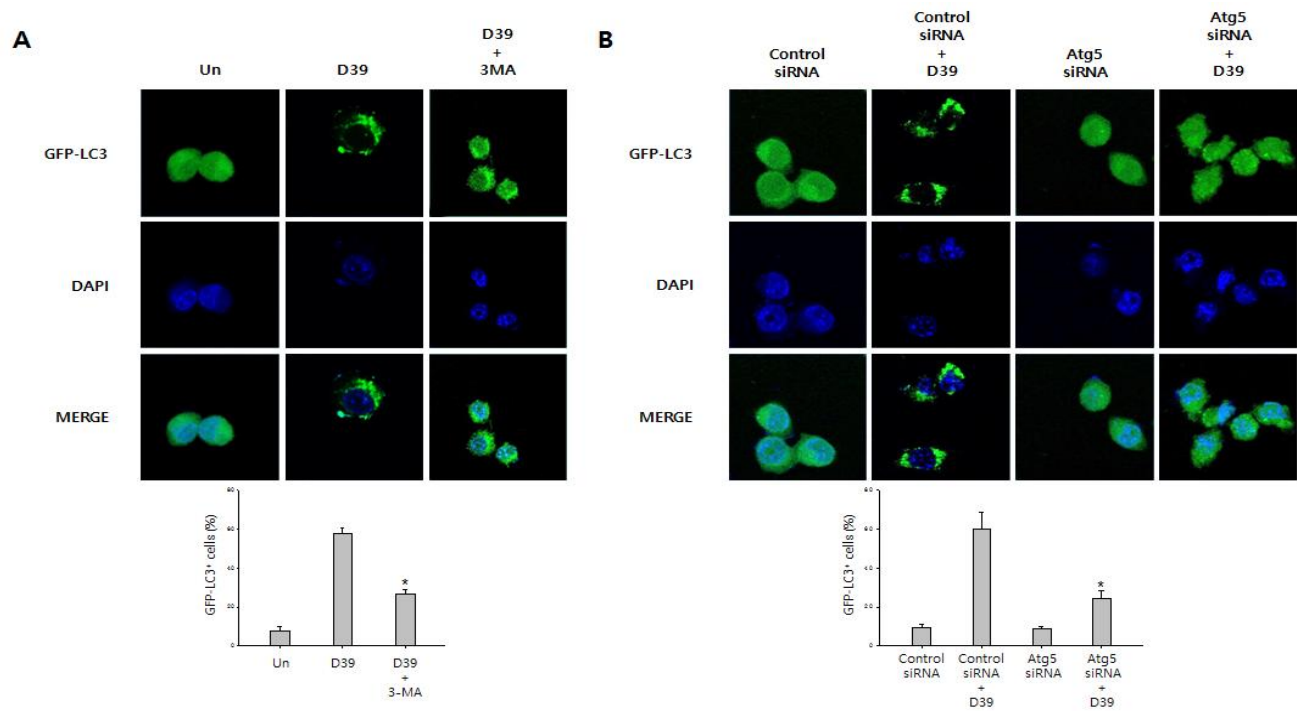
Supplementary Figure S2: *S. pneumoniae* induces the production of IL-1 β , IL-6 and TNF- α . BV-2 microglial cells were infected with D39 (A) or TIGR4 (B) at an MOI of 100 for 4 h and 8 h. The levels of IL-1 β , IL-6 and TNF- α in the culture supernatants were determined by ELISA. Data are expressed as the mean \pm S.E.M. of 3 experiments. * $p < 0.05$, significantly different from uninfected control. [#] $p < 0.05$, Statistical difference with respect to time of pneumococcal infection.



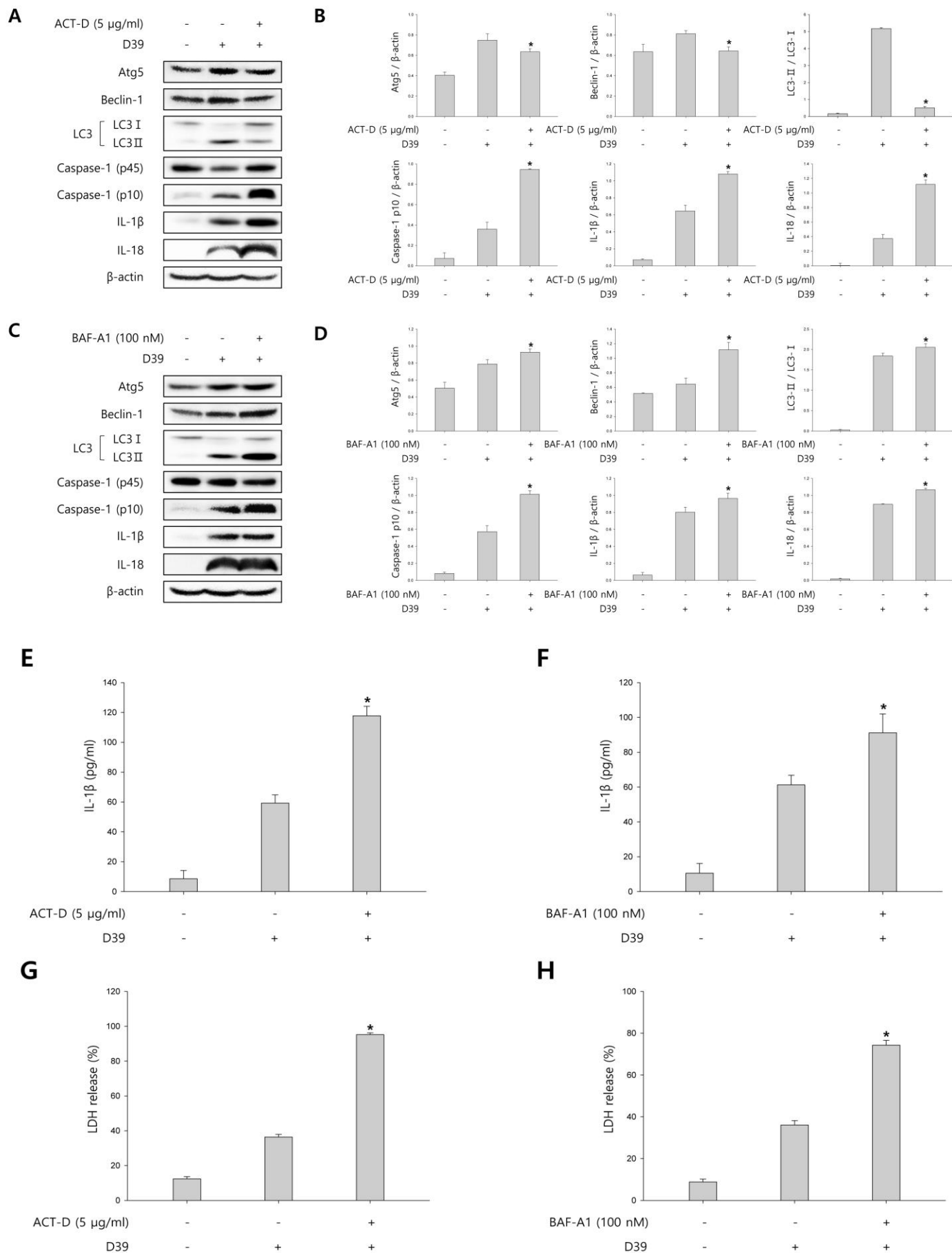
Supplementary Figure S3: *S. pneumoniae* D39 induces caspase-1 activation and pyroptosis in C6 glioma cells. (A-D) C6 glioma cells were left uninfected or were infected with *S. pneumoniae* D39 at various MOI of 25, 50 and 100 for 1, 2 and 4 h. Cell lysates were subjected to Western blot analysis to detect caspase-1, IL-1 β and IL-18. The levels of each protein expression are in arbitrary units, and data are normalized to the respective amount of β -actin protein. These results are representative of three independent experiments with similar results. * $p < 0.05$, significantly different from uninfected control. (E) C6 cells were left uninfected or were infected with D39 at an MOI of 100 for 4 h. Culture supernatants were concentrated by trichloroacetate precipitation. Concentrated supernatants and cell lysates were subjected to Western blot analysis using antibodies specific for caspase-1, IL-1 β , IL-18 and β -actin. β -actin was used as a loading control. These results are representative of three independent experiments. (F) C6 glioma cells were infected with D39 at an MOI of 100 for 4 h and 8 h. LDH release into the culture media is shown as a percentage of LDH release. Data are expressed as the mean \pm S.E.M. of 3 experiments. *Significantly different from uninfected cells ($P < 0.05$).



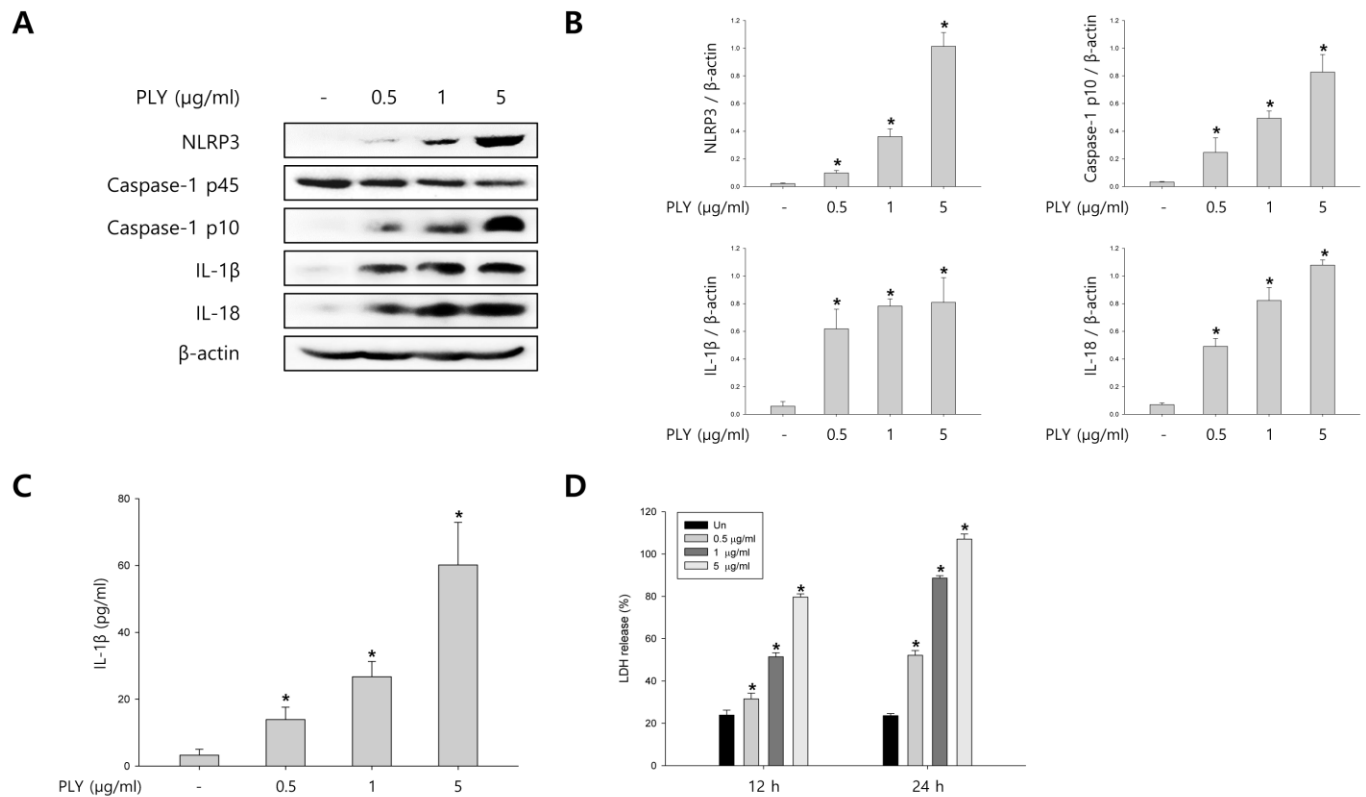
Supplementary Figure S4: Time-dependent expression of inflammasome-related proteins, caspase-1, caspase-1-dependent cytokines and autophagy-related proteins. BV-2 microglial cells were infected with D39 at an MOI of 100 for indicated times. Cell lysates were subjected to Western blot analysis. β-actin was used as a loading control. These results are representative of three independent experiments with similar results. *p < 0.05, significantly different from uninfected control.



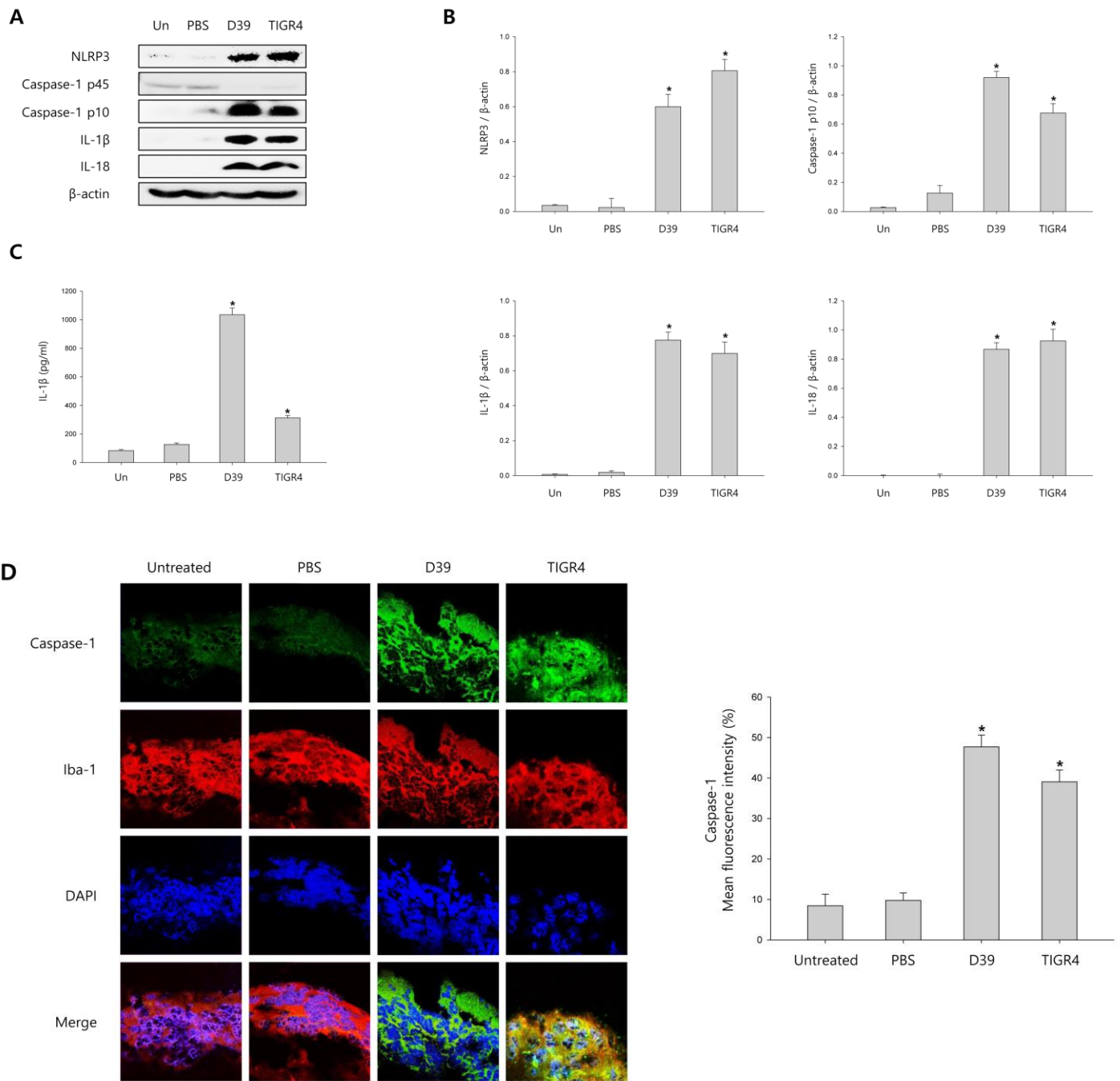
Supplementary Figure S5: Autophagy is induced by *S. pneumoniae*. (A) BV-2 cells were transfected with GFP-LC3 for 24 h and infected with *S. pneumoniae* D39 in the presence or absence of 3-MA (1 mM) at an MOI of 100 for 4 h. (B) BV-2 cells were co-transfected with GFP-LC3 and siRNA (control siRNA or Atg5 siRNA) for 24 h and infected with *S. pneumoniae* D39 at an MOI of 100 for 4 h. GFP-LC3 expressing cells were visualized with a fluorescence microscope and the results are presented as quantification of GFP-LC3⁺ dots. These results are representative of three independent experiments with similar results. * $p < 0.05$, significantly different from *S. pneumoniae* D39-infected control.



Supplementary Figure S6: Autophagy inhibits *S. pneumoniae*-induced caspase-1 activation and pyroptosis. (A and B) BV-2 cells were left uninfected or were infected with D39 at an MOI of 100 for 2 h (Atg5 and Beclin-1) and 4 h in the presence or absence of actinomycin D (ACT-D, 5 µg/ml). Cell lysates were subjected to Western blot analysis. The levels of each protein expression are in arbitrary units, and data are normalized to the respective amount of β-actin protein. The LC3 I and LC3 II bands were quantified by densitometry. These results are representative of three independent experiments with similar results. *p < 0.05, significantly different from *S. pneumoniae* D39-infected control. (C and D) BV-2 cells were left uninfected or were infected with D39 at an MOI of 100 for 2 h (Atg5 and Beclin-1) and 4 h in the presence or absence of bafilomycin A1 (BAF-A1, 100 nM). Cell lysates were subjected to Western blot analysis. The levels of each protein expression are in arbitrary units, and data are normalized to the respective amount of β-actin protein. The LC3 I and LC3 II bands were quantified by densitometry. These results are representative of three independent experiments with similar results. *p < 0.05, significantly different from *S. pneumoniae* D39-infected control. (E and G) BV-2 microglial cells were left uninfected or were infected with D39 at an MOI of 100 for 8 h in the presence or absence of actinomycin D (ACT-D, 5 µg/ml). (F and H) BV-2 cells were left uninfected or were infected with D39 at an MOI of 100 for 8 h in the presence or absence of bafilomycin A1 (BAF-A1, 100 nM). The levels of IL-1β in the culture supernatants were determined by ELISA (E and F). The levels of LDH in the culture media are shown as a percentage of LDH release (G and H). These results are representative of three independent experiments with similar results. *p < 0.05, significantly different from *S. pneumoniae* D39-infected control.



Supplementary Figure S7: Pneumolysin induces caspase-1 activation and triggers pyroptosis. (A and B) BV-2 microglial cells were treated with 0.5, 1 and 5 µg/ml of pneumolysin (PLY) for 6 h (NLRP3) and 12 h. Cell lysates were subjected to immunoblot analysis. The levels of each protein expression are in arbitrary units, and data are normalized to the respective amount of β-actin protein. These results are representative of three independent experiments with similar results. * $p < 0.05$, significantly different from untreated control. (C) BV-2 cells were treated with 0.5, 1 and 5 µg/ml of PLY for 24 h. The levels of IL-1β in the culture supernatants were determined by ELISA. These results are representative of three independent experiments with similar results. * $p < 0.05$, significantly different from untreated control. (D) Microglial cells were treated with 0.5, 1 and 5 µg/ml of PLY for 12 h and 24 h. The levels of LDH in the culture supernatant are shown as a percentage of LDH release. These results are representative of three independent experiments with similar results. * $p < 0.05$, significantly different from untreated control.



Supplementary Figure S8: *S. pneumoniae* induces caspase-1 activation in murine pneumococcal meningitis. (A and B) C57BL/6 mice were challenged with control PBS, *S. pneumoniae* D39 or *S. pneumoniae* TIGR4 via intracerebral ventricular injection. The protein expression of NLRP3, Caspase-1, IL-1 β and IL-18 in brain homogenates was determined by Western blot analysis. The levels of each protein expression are in arbitrary units, and data are normalized to the respective amount of β -actin protein. These results are representative of three independent experiments with similar results. * $p < 0.05$, significantly different from PBS-treated control. (C) The levels of IL-1 β in brain homogenates were determined by ELISA. These results are representative of three independent experiments with similar results. * $p < 0.05$, significantly different from PBS-treated control. (D) Immunohistochemical analysis of caspase-1 in *S. pneumoniae*-infected mouse brains. Sections were stained for caspase-1 (FITC) and the glial cell marker, Iba-1 (Texas Red) (x400).